Neuron, Vol. 14, 407–412, February, 1995, Copyright © 1995 by Cell Press

Persistent Activation of Min K Channels by Chemical Cross-Linking

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Summary

Expression of the structurally and functionally distinct min K channel in Xenopus oocytes results in voltage-dependent potassium currents that activate with a characteristic slow time course. Application of a membrane-impermeable chemical cross-linking agent to oocytes expressing min K decreased the time-dependent current, increased its rate of activation, and induced persistently activated inward and outward potassium currents. These effects required membrane depolarization, demonstrating use dependence. Persistently activated channels retained potassium selectivity and sensitivity to block by clofilium and barium. These results suggest that a major conformational change occurs during min K channel gating, which can be stabilized by chemical cross-linking, and are consistent with a model in which min K channels activate by voltage-dependent subunit aggregation.

Introduction

One voltage-dependent potassium channel, min K or I_{sK}, is structurally and functionally distinct. The min K protein contains only 130 amino acids and a single predicted transmembrane domain. Min K displays no homology with other cloned potassium channels (Takumi et al., 1988), although it shares an overall topology with two other slowly activating channels, phospholemman, a chloride channel (Moorman et al., 1992), and the influenza M_2 protein, which is permeable to protons (Pinto et al., 1992). When expressed in Xenopus laevis oocytes (Takumi et al., 1988; Busch et al., 1992) or HEK 293 cells (Freeman and Kass, 1993), the min K protein induces voltage-dependent, potassium-selective currents that activate slowly, on the order of seconds or tens of seconds. Even in response to voltage commands of several minutes in duration, these currents do not reach a steady state, but continue to grow in amplitude. The slow kinetics of min K gating and the lack of homology with other voltage-gated potassium channels suggest that min K channels may activate by a fundamentally different mechanism. However, mutagenesis of the min K protein altered its activation kinetics (Takumi et al., 1991), and mutations within the transmembrane domain altered ion selectivity (Goldstein and Miller, 1991), supporting the hypothesis that the min K protein is itself a structural component of a potassium channel. To probe the gating mechanism of min K, we now report the results of experiments in which a chemical cross-linking agent was applied to min K–expressing oocytes.

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Results

DTSSP Induces Instantaneous Currents and Alters Time-Dependent Currents

Rat min K, engineered to include the FLAG epitope at the C-terminus and expressed in Xenopus oocytes, generated the characteristic, slowly activating outward current at potentials positive to −60 mV. Application of 1.5 mM 3,3-dithio-bis(sulfosuccinimidyl proprionate) (DTSSP) for 15–20 min, with concurrent 20 s membrane depolarizations every minute, progressively reduced deactivation, producing persistent tail currents at −60 mV; increased the instantaneous current observed at the beginning of the test pulse; decreased the time-dependent component of the outward current; and altered its activation kinetics (Figures 1A and 1B). These changes were accompanied by a decrease in the oocyte input resistance (Figure 1B) and an increase in the holding current at −80 mV (data not shown). The effect of DTSSP persisted even after prolonged washout (Figures 1A and 1B, trace d).

The current–voltage relation and kinetics of activation and deactivation of the modified currents were examined. The family of current records demonstrated that chemical cross-linking induced currents at potentials negative to the activation threshold for untreated min K channels (Figures 2A and 2B). Little or no time-dependent current was observed at potentials between −120 and −80 mV, whereas at more depolarized potentials, an additional time-dependent component remained. However, the voltage dependence of this component was altered by DTSSP (Figure 2C); applying the Boltzmann function indicated that the $V_{½}$ shifted −29.0 ± 1.1 mV (n = 11), and the slope factor (k) increased from 12.6 ± 0.3 (n = 15) to 16.2 ± 0.7 (n = 11; Table 1). In addition, the time course for activation of the time-dependent component was faster following DTSSP treatment and lacked the characteristic sigmoidal activation kinetics (Figure 2B). A sum of two exponentials fitted to the delayed increase in current at 20 mV showed that τ_{fast} decreased from 7.7 ± 0.5 to 1.9 ± 0.1 s, τ_{slow} decreased from 24.6 ± 1.3 to 13.6 ± 0.6 s, and the fractional amplitude of the fast component, $A_{fast}/(A_{fast} + A_{slow})$, decreased from 0.38 ± 0.02 to 0.23 ± 0.01 after DTSSP treatment (Table 2; n = 28). In contrast, the kinetics of the deactivating tail currents were only slightly altered. A single exponential fit to deactivation yielded a time constant that changed from 2.2 ± 0.1 to 2.4 ± 0.1 s.

Some relaxation of the persistently activated min K currents was observed during long pulses to very hyperpolarized potentials (Figure 2B, bottom trace). Associated with this relaxation was a small decrease in the instantaneous current apparent in the following depolarizing pulse and an increase in the input resistance (data not shown). Both

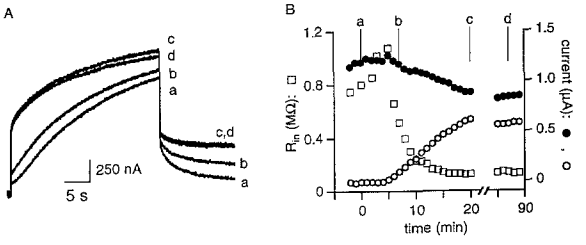

Figure 1. Chemical Modification of Min K Currents by DTSSP

(A) DTSSP (1.5 mM) was applied for 20 min in the bath solution during repetitive 20 s depolarizing pulses to 20 mV made every minute. Shown are representative current traces elicited before, during, and after application and washout of DTSSP; a, time 0; b, 7 min; c, 20 min; d, 87 min. A 500 ms prepulse to −100 mV from a holding potential of −80 mV was used; tail currents were recorded at −60 mV. Time scale bar is positioned at 0 current.

(B) Time course of the DTSSP effect on oocyte input resistance (open squares) and instantaneous (open circles) and time-dependent (closed circles) min K currents for 20 min application of cross-linking agent and subsequent washout. DTSSP was added at time 0; letters correspond to labeled traces in (A).

Table 1. DTSSP Alters the Voltage Dependence for Activation of the Time-Dependent Component of Min K Currents

Group	G_{max} (% control)	$\Delta V_{½}$ (mV)	k (mV)	n
Control	100		12.6 ± 0.1	15
DTSSP	66 ± 2*	−29.0 ± 1.1*	16.2 ± 0.7*	11
Sulfo-NHS-acetate	119 ± 5*	−2.8 ± 1.1	13.2 ± 0.8	4

Values represent mean ± SEM. Parameters were determined by fitting conductance–voltage data (from 20 s test pulses) with a Boltzmann relation using a Levenberg–Marquardt algorithm to minimize the sum of squares.

* $p < .05$ for respective paired control values determined by a t test.

the instantaneous current and the input resistance recovered fully during subsequent depolarizations, without a change in the size or kinetics of the time-dependent outward current. The relaxation of inward currents at −140 mV was not dependent upon external magnesium or calcium (data not shown).

The initial time course of DTSSP-modified instantaneous currents was examined at a faster sampling rate during 50 ms voltage steps. Only negligible currents were observed in untreated min K–expressing oocytes, as expected for its slow rate of activation (Figure 2D). In contrast, DTSSP treatment induced significant outward as well as inward currents during 50 ms pulses and revealed no kinetics following the capacitive transient (Figures 2E and 2F). The nearly linear current–voltage relation reflected the apparent loss of voltage- and time-dependent gating, indicating persistent activation of the channels. Some rectification, however, was present at more extreme potentials. In addition, instantaneous currents reversed at −91 ± 2.5 mV (n = 8), near the expected potassium reversal potential for oocytes under these ionic conditions (n = 13). These results suggest that cross-linking of min K holds the channel in an open state.

The effects of DTSSP were specific for oocytes expressing min K. Noninjected oocytes displayed small currents that were reduced or unchanged by DTSSP (Figure 2F). Application of DTSSP to oocytes expressing the *Shaker*-like potassium channel RBK1 (Christie et al., 1989) resulted in no increase in the instantaneous current, no obvious change in the kinetics of activation, and only a small decrease in the time-dependent outward current (n = 5).

DTSSP-Treated Channels Retained Potassium Selectivity and Pharmacology

The modified currents retained many of the essential attributes of min K currents. Changing the external potassium

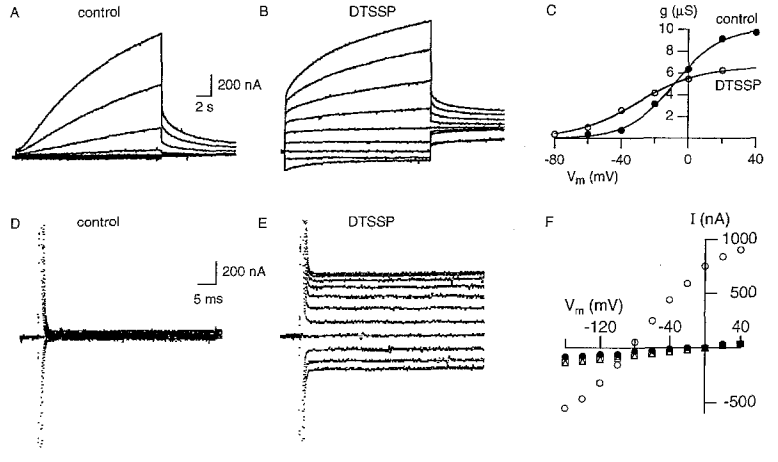

Figure 2. Effect of DTSSP on Current–Voltage Relation of Min K

(A and B) Representative family of current traces elicited by 20 s voltage commands from a prepulse potential of −100 mV to potentials from −140 to 20 mV in 20 mV steps, before (A) and after (B) 20 min application and washout of 1.5 mM DTSSP. Tail currents measured at −60 mV.

(C) Conductance–voltage relation for the time-dependent component of min K currents (from [A] and [B]) before (closed circles) and after (open circles) modification by DTSSP. Continuous curves were drawn according to a Boltzmann function.

(D and E) Examination of control (D) and DTSSP-modified (E) currents at a faster sampling rate (20 kHz) evoked by 50 ms voltage commands to potentials from −160 to 40 mV in 20 mV steps.

(F) Current–voltage relation of 50 ms traces for a min K–expressing oocyte before (closed circles) and after (open circles) DTSSP treatment, and for a representative noninjected oocyte before (open triangles) and after (open circles) DTSSP. In both cases, DTSSP was applied during repetitive 20 s depolarizations to 20 mV made every minute.

Table 2. DTSSP Alters the Kinetics of the Time-Dependent Component of Min K Currents

Group	Activation			Deactivation	
	τ_{fast}	τ_{slow}	$A_f/(A_f + A_s)$	τ	n
Control	7.7 ± 0.5 s	24.6 ± 1.3 s	0.38 ± 0.02	2.2 ± 0.1 s	28
DTSSP	1.9 ± 0.1 s	13.6 ± 0.6 s	0.23 ± 0.01	2.4 ± 0.1 s	28

The kinetics of activation were determined by fitting a sum of two exponentials to the delayed increase in current at 20 mV. The kinetics of deactivation were determined using a single exponential fit to the tail currents at −60 mV. Values represent the mean ± SEM.

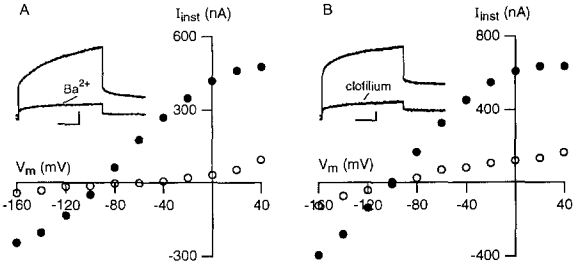

Figure 3. Pharmacology of DTSSP-Modified Min K Channels

(A) Barium block of DTSSP-modified min K channels. Initial current–voltage relation for 50 ms voltage commands from -160 to 40 mV, before (closed circles) and after (open circles) 20 mM barium. The inset shows current traces for DTSSP-modified min K channels for 20 s depolarizations to 20 mV, before and after external application of 20 mM barium. Bars, 150 nA and 5 s.

(B) Clofilium block of DTSSP-modified min K currents. Initial current–voltage relation for 50 ms voltage commands from −160 to 40 mV, before (closed circles) and after (open circles) 100 μM clofilium. The inset shows current traces for DTSSP-modified min K channels for 20 s depolarizations to 20 mV, before and after external application of 100 μM clofilium. Bars, 150 nA and 5 s.

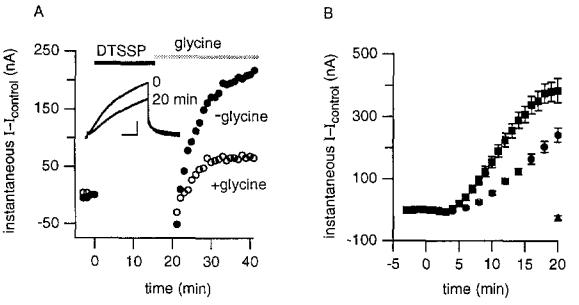

Figure 4. Chemical Modification of Min K Currents Required Channel Opening

(A) Time course of increase in the instantaneous current with repetitive depolarization initiated after 15 min exposure and 5 min washout of 1.5 mM DTSSP; the membrane potential was held at −100 mV during this time. The mean increase in the instantaneous current under these conditions (closed circles) was 200 ± 24 nA (n = 5); this increase was attenuated by 20 mM glycine in the wash (open circles; +73.8 ± 36 nA; n = 5; $p < .05$). The inset shows current traces for a representative oocyte before (0) and after 15 min application and 5 min washout (20 min) of 1.5 mM DTSSP. Bars, 500 nA and 5 s.

(B) Use dependence of the effect of DTSSP on the instantaneous min K current. Membrane depolarizations for 20 s to 20 mV were made every 1 min (closed squares; n = 33–45), every 2 min (closed circles; n = 4), or after 20 min (closed triangle; n = 11) during application of 1.5 mM DTSSP. The slope of a line fit to the average increase over control in the instantaneous current was 29 nA/min (r = 0.997) and 17 nA/min (r = 0.997) for depolarizations every 1 and 2 min, respectively.

concentration from 2 to 20 mM shifted the reversal potential of the persistently activated currents +56.2 ± 1.6 mV (n = 8), in agreement with that predicted by the Nernst equation for potassium. In contrast, removal of external chloride had little effect on the reversal potential of persistently activated currents, shifting V_{rev} only −0.8 ± 4.6 mV (n = 4). External 20 mM barium reduced both instantaneous and time-dependent cross-linked currents by 92% ± 3% and 81% ± 2% (n = 6), respectively; both inward and outward currents were blocked (Figure 3A). The dose–response for inhibition of currents during 50 ms steps to 0 mV gave a K_D value of 0.33 ± 0.07 mM (n = 4); from the voltage dependence of the K_D value, a δ value of 0.17 was obtained (Woodhull, 1973; Hausdorff et al., 1991). Clofilium, a class III anti-arrhythmic drug known to inhibit min K currents (Folander et al., 1990; Honoré et al., 1991; Varnum et al., 1993), also reduced DTSSP-modified currents (Figure 3B); the instantaneous component was inhibited 78% ± 4.5%, and the time-dependent component was inhibited 63% ± 3.0% (n = 9). Clofilium blocked inward currents nearly as effectively as outward currents. These results indicate that, although gating was altered dramatically by DTSSP, the fundamental pore properties of min K channels (Goldstein and Miller, 1991; Hausdorff et al., 1991) were not changed significantly.

Persistent Activation Requires Membrane Depolarizations

When the membrane potential was held at −100 mV during application of DTSSP, the first depolarization to 20 mV after washout revealed no persistently activated current (Figure 4A). However, continued depolarizations at 1 min intervals produced some increase in the instantaneous current. This increase was inhibited by addition of 20 mM glycine in the wash, which provides a primary amine group that competes for DTSSP (Figure 4A). The use dependence of the cross-linking effect was demonstrated further by varying the frequency of depolarizing commands during DTSSP application. As shown in Figure 4B, the rate of change in the instantaneous current was proportional to the frequency of membrane depolarizations. These results demonstrate that min K channels need to be activated for DTSSP to exert its effects.

Cross-Linking Effects Require Bifunctional Acylation

As does DTSSP, sulfo-NHS-acetate acylates amino groups,

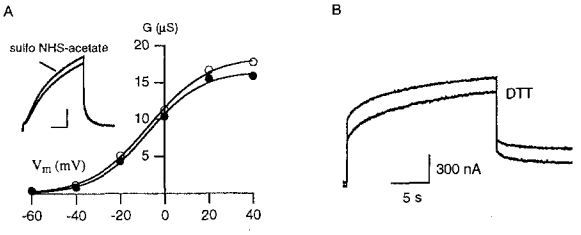

Figure 5. Modification of Currents Required Bifunctional Acylation

(A) Effect of the monofunctional acylating agent, sulfo-NHS-acetate, applied under conditions identical to those used for DTSSP. The inset shows current traces for a representative oocyte before and after 20 min application and washout of 1.5 mM sulfo-NHS-acetate. Conductance–voltage relation for the time-dependent component of min K currents before (closed circles) and after (open circles) treatment with the monofunctional compound. Continuous curves were drawn according to a Boltzmann function. Bars, 500 nA and 5 s.

(B) Chemical reduction of cross-linked channels; 5 mM dithiothreitol (DTT) was applied for 15 min to DTSSP-treated oocytes during repetitive 20 s depolarizations to 20 mV.

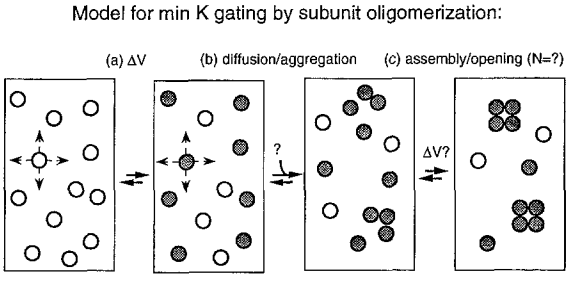

Figure 6. Model for Min K Channel Gating by Subunit Oligomerization

Open circles represent nonactive min K subunits freely diffusing in the plane of the membrane. Closed circles represent min K subunits whose conformation has been altered by depolarization (ΔV), and are therefore able to aggregate and assemble into functional pores of unknown stoichiometry (N). Complete channel complexes that have been tethered together by DTSSP remain persistently activated. Dimers, trimers, etc. activate with addition of other subunits, at a more rapid rate than untreated subunits alone in the fully deactivated state; these deactivate at a rate similar to untreated channels, with the loss of only one untethered subunit.

but it adds an acetyl group that cannot react further; DTSSP can react with two amines, adding a dithiobis(propionyl) group between them. No cross-linking effect was observed when sulfo-NHS-acetate was applied under conditions identical to those used for DTSSP (Figure 5A). The time-dependent outward current was increased 11.1% ± 2.3% (n = 7), but its voltage dependence was not altered significantly (Table 1). In contrast to the use dependency of the DTSSP effect, there was little difference seen between application of sulfo-NHS-acetate during repetitive depolarizations or while channels were held closed at hyperpolarized potentials (n = 5).

DTSSP contains a disulfide bond centered within its symmetrical 12 Å spacer arm. The reducing agent dithiothreitol (DTT; 5–10 mM) was applied briefly subsequent to DTSSP application and washout. After DTT, depolarizing commands evoked currents that were reduced in amplitude compared with currents before DTT application (Figure 5B). The instantaneous current was reduced by 35% ± 6% (n = 5), but the time-dependent component was altered only slightly (−7.7% ± 5%). Thus, cross-linked channels may be partially sensitive to disruption of the cross-linking agent. Application of higher concentrations of DTT (25–50 mM) or longer exposure (30–60 min) resulted in nonviable oocytes. Thus, it was not possible to reverse the effect of DTSSP fully, owing to the harsh conditions required for complete reduction of its disulfide bonds. These results confirm that bifunctional conjugation is necessary for the cross-linking effect on min K currents.

Discussion

Application of a chemical cross-linking agent, DTSSP, to min K–expressing oocytes resulted in a population of channels that lost voltage-dependent gating, yet retained the fundamental properties of ion selectivity and sensitivity to specific blocking agents. These results show that the voltage-dependent gating of min K channels is profoundly altered by chemical cross-linking, and that a major conformational change is involved with min K channel opening. The results provide no direct evidence for an aggregation mechanism of channel gating, such as for alamethicin; any model in which a major conformational change is involved in channel opening can be accomodated. Indeed, the effects of DTSSP may be due to intrasubunit cross-linking or to linkage by only one end of DTSSP. The results are, however, consistent with an aggregation model (Figure 6) (Baumann and Mueller, 1974).

The current traces obtained after cross-linking reflect a mixed population of channel molecules. One population consists of persistently activated channels and may be due to channels locked in the open state by intersubunit cross-links. A second population of channels is apparent only under depolarizing conditions. These activate in a time- and voltage-dependent manner and do not reach steady state even during long depolarizing commands; as expected, this population is not seen at potentials negative to the normal threshold of activation. However, the activation kinetics of this population are speeded, and this may be due to cross-linked complexes of less than functional numbers of subunits. If activation were dependent upon subunit aggregation, then this "partially activated" population would require fewer than the functional number of productive subunit collisions, hence activating more rapidly. The time constants of the tail currents are not altered significantly following DTSSP treatment, suggesting that deactivation proceeds by the reverse process, the uncoupling of one or more subunits. A third population of subunits is revealed by the decrease in the current amplitude following DTSSP application in the absence of depolarizing commands, resulting in a reduced time- and voltage-dependent current amplitude, but not persistently activated channels. This may be due to subunits that have been bound by one end of the homobifunctional cross-linker, but have not been cross-linked to another subunit on the other end and are thus less able to integrate into

functional channel complexes, perhaps through steric hindrance.

The effects of DTSSP were dependent upon channel opening and were not reversed by prolonged washout, consistent with a covalent modification of the channel protein. This suggests that a major conformational change occurs during channel opening, which can be stabilized by chemical cross-linking. A related but monofunctional acylating agent, sulfo-NHS-acetate, did not mimic the effect of DTSSP, indicating that the bifunctional structure of DTSSP was crucial to its modification of min K currents. Furthermore, DTSSP had no effect on oocytes that were not previously injected with min K mRNA and little effect on oocytes expressing the *Shaker*-like potassium channel, RBK1 (Kv1.1). Activation of min K may thus involve a mechanism fundamentally different from that of *Shaker*-like channels.

DTSSP may chemically modify channel surface electrostatics, as it would be expected to abolish the charges of participating amino acid side chains. This is unlikely to explain persistent activation of min K currents, since sulfo-NHS-acetate was unable to enhance channel activity. Modification of residues exposed to the extracellular solution could, however, be responsible for the change in amplitude of time-dependent currents seen following simple acylation by the monofunctional agent or after application of the cross-linking agent while channels were held closed. Alternatively, these conditions could affect ion permeation by subtly altering properties of the pore.

The action of DTSSP on min K–induced currents may provide important clues regarding the gating mechanism for this channel. Differences seen between the effect of the cross-linking agent on expressed channels with versus without depolarization may reflect dissimilar exposure of reactive sites in the extracellular domain of min K or varying distances separating reactive sites in open versus closed configurations. In the first case, intrasubunit cross-linking of open channels may hold an activation gate open. In the latter case, channel activation may involve subunit oligomerization, and the proximity of subunits would then permit DTSSP to grapple channel subunits together, thus favoring the conducting conformation and inhibiting deactivation (deoligomerization). This latter model for channel gating could account for the remarkably slow activation kinetics of min K currents and the susceptibility of the activation kinetics to changes in subunit density and temperature (unpublished data). It also offers a reasonable explanation for how a simple protein with only one putative transmembrane domain could form an ion-conducting pathway—by aggregation of multiple, diffusing subunits.

Although we interpret the results in terms of a model in which min K channel subunits activate by subunit aggregation, they may be the result of an indirect effect of DTSSP on min K currents, acting via an endogenous membrane protein, or through an allosteric effect by which the min K protein regulates an endogenous channel (Attali et al., 1993); such interactions might also be stabilized by chemical cross-linking. Indeed, the results with DTSSP are consistent with subunit oligomerization, whatever the basis for the channel activity induced by expression of the min K protein may be.

Experimental Procedures

Oligonucleotide-directed mutagenesis, in vitro mRNA synthesis, and oocyte injection and handling were performed as previously described (Adelman et al., 1992). Xenopus care and handling were in accordance with the highest standards of institutional guidelines. Frogs underwent no more than two surgeries, separated by at least 3 weeks, and surgeries were performed using well established techniques by experienced members of the Animal Care staff. Frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester. Xenopus oocytes were injected with 0.5 ng of capped mRNA; 2–7 days after injection, macroscopic currents were measured using a two-electrode voltage clamp with a TEV-200 or CA-1 amplifier (Dagan Corporation, Minneapolis, MN) interfaced to a LSI 1173 computer. Oocytes were superfused continuously (1–2 ml/min) with ND-96 containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.6) at room temperature (21°C–23°C). Experiments were generally performed with the oocyte membrane held at −80 or −100 mV. The general properties of the min K–FLAG currents did not differ substantially from those of wild-type rat min K. Values from experiments with multiple data points are presented as the mean ± SEM. Modifying agents and drugs were applied externally in ND-96. Chemical modifying agents DTSSP and sulfo-NHS-acetate (sulfosuccinimidyl acetate) were from Pierce (Rockford, IL). Neither chemical agent substantially altered the pH of the perfusing solution (pH 7.6). Because the activity of these compounds varied with lot number, the general reactivity of the lots used in these experiments was confirmed by monitoring hydrolysis in ND-96, as indicated by the increase in absorbance at 260 nm caused by release of the N-hydroxysuccinimide group (Abdella et al., 1979). Clofilium was from Research Biochemicals (Natick, MA); DTT was from Sigma (St. Louis, MO).

Acknowledgments

All correspondence should be addressed to J. P. A. We thank our colleagues Dr. Bill Groh and Thanos Tzounoupolis for stimulating discussions. This work was supported by NIH grants to J. M. and J. P. A., and in part by a Tartar Fellowship Award to M. D. V.

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Received August 4, 1994; revised October 5, 1994.

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